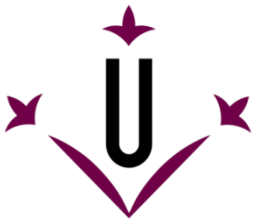

Title: Formation of Patulin-Glutathione conjugates induced by pulsed light: a tentative strategy for patulin degradation in apple juices**.**

Authors: Juan José Rodríguez-Bencomo*; Vicente Sanchis; Inmaculada Viñas; Olga Martín-Belloso; Robert Soliva-Fortuny

Address: Food Technology Department, UTPV-XaRTA, Agrotecnio Center, University of Lleida, Rovira Roure 191, 25198, Lleida, Spain**.**

Running title: Patulin-Glutathione conjugates for degrading patulin in apple juices

*corresponding author: jrbencomo@gmail.com / pvrodriguez@tecal.udl.cat

Abstract

Patulin is a toxic mycotoxin usually associated with apple products. Due to its unhealthy effects for humans, its content is regulated by the food safety authorities. The removal or degradation of this mycotoxin in contaminated apple juices has been studied with different approaches with uneven effectiveness. However, a strategy based on the chemical reaction between patulin and glutathione (GSH), in order to generate the conjugates that are formed during cell detoxification process, is an innovative approach yet to be evaluated. In this work, the formation of patulin-GSH conjugates activated by the application of pulsed light treatments and catalyzed by Fe^{+2} ions was evaluated. The study of patulin degradation and effect of the GSH/Fe^{+2} molar ratio showed that a molar ratio of 5 allows an adequate catalytic effect of the metal ions. In addition, mono-substituted patulin-glutathione adducts were identified as the main type of generated conjugates.

Keywords: Patulin, Apple juice, Detoxification, Patulin-Glutathione adducts, Pulsed light.

1. Introduction.

Apple products are considered to be by far the most significant dietary source of patulin. Its origin is related with the action of molds, mainly *Penicillium expansum*. In humans, the unhealthy effects of this mycotoxin include gastrointestinal disorders, nausea and vomiting, although there is no evidence of chronic effects (Puel, Galtier & Oswald, 2010; Ioi, Zhou, Tsao & Marcone, 2017). Based on its toxicity, the European Union (EU) has established a maximum content of 50 and 25 µg/Kg of patulin for apple-based juices and solid food products, respectively. In the case of products labeled as baby foods the established limit is 10 µg/Kg (CE No 1881/2006). However, even in countries with regulated levels and despite the efforts of juice processors to control the quality of the raw materials, it is possible to detect samples in the marked with levels slightly higher than the maximum allowed (Zhong, Carere, Lu, Lu, & Zhou, 2018).

Consequently, fruit producers, food processors and researchers have focused their efforts on the search for strategies to mitigate the contamination of patulin from the raw materials to the end products. However, as pre-harvest treatments do not always ensure enough low levels of patulin in the juices, different approaches have been investigated in order to remove or degrade this toxin during food processing (Ioi et al., 2017). In this sense, usual processing techniques such as the addition of enzymes and clarifying agents have been reported to produce uneven removal effectiveness and non-desired losses of other components (Bissessur, Permaul & Odhav, 2001; Gökmen, Artık, Acar, Kahraman & Poyrazoglu, 2001). Filtration or ultra-filtration processes have shown very limited patulin removal effectiveness (Acar, Gökmen & Taydas, 1998; Welke, Hoeltz, Dottori & Noll, 2009). As well, thermal treatments, such as pasteurization, have also shown limited patulin degradation effectiveness. Furthermore, intense thermal treatments negatively affect the organoleptic characteristics of juices (Kadakal & Nas, 2003).

Over the last decades, novel non-thermal food processing techniques have been developed as an alternative to the classical thermal processes. These non-thermal techniques were initially devised to ensure the microbiological safety and stability of food products minimizing the limitations of thermal treatments related to the effects on the organoleptic and nutritional characteristics of the products (Zhang, Wang, Zeng, Han & Brennan, 2019). Some of these novel techniques have been assayed for degrading patulin in apple juices. Avsaroglu, Bozoglu, Alpas, Largeteau and Demazeau (2015) studied the application of high hydrostatic pressure processing (pulsed or continuous) and its effects on patulin content, obtaining degradation up to 62 % but only for low initial patulin contents (5 µg/L). The effectiveness of high pressure processing on patulin degradation has been shown to depend on the juice composition, particularly, on the presence of compounds containing thiol groups (Hao, Zhou, Koutchma, Wu & Warriner 2016). Treatments based on irradiation with light have been also assayed. Zhu, Koutchma, Warriner and Zhou (2014) applied ultraviolet (UV) light to contaminated apple juices, finding that patulin degradation effectiveness depended on the wavelength, obtaining 90% of degradation at 222 nm with non significant changes in color. Pulsed white light treatments have been as well studied for patuline degradation. Funes, Gómez, Resnik and Alzamora (2013) obtained degradation of up to a 78 % of the initial content in apple juices by using pulsed light with a total energy dose of 35.8 J/cm^2. Although these works are promising regarding the effectiveness of patulin degradation, especially in the case of light treatments, none of them determined the type of degradation products generated, which is a limiting aspect for its application in food products.

Another approach is the addition or supplementation with chemical compounds that, under specific conditions, can cause patulin degradation. Thereby, the addition of ascorbic acid allowed degrading up to 70 % of patulin in a model juice solution after 34 days (Drusch, Kopka & Kaeding, 2007). However, these authors concluded that the presence of oxygen and free radicals is necessary for a rapid degradation, which limits its application in real juice. Other

78 authors have investigated the possible formation of adducts (or conjugates) with sulfur
79 compounds such as thiols (Merkulow & Ludwig, 2002; Folger, 2014), which can be naturally
80 present in the juices or be supplemented (Jones et al., 1992), being this approach based on the
81 chemical behavior of patulin into the cells. Thus, the electrophilic character of the patulin
82 molecule allows its reaction with nucleophiles (such as thiol groups), inhibiting the action of
83 numerous enzymes and binding with other important cell components such as DNA, peptides
84 (e.g. glutathione (GSH)) and aminoacids (e.g. cysteine) (Pal, Singh & Ansari, 2017). These
85 reactions occur in the complex cell media in which enzymes and other substances can
86 intervene. For instance, the formation of GSH adducts is produced by enzymatic pathways
87 (Sheehan, Meade, Foley & Dowd, 2001), and for the reactions with DNA, a role of divalent
88 metal ions (in presence of a reducing agent) has been hypothesized (Lee & Roschenthaler,
89 1987).

90 Regarding the toxic effects of these thiol-patulin adducts, Lindroth and Von Wright (1978)
91 compared the toxicity of patulin-cysteine adducts in mice, obtaining 100-times lower toxicity
92 for adducts than for free patulin; however, teratogenicity to chicken embryos was retained
93 (Ciegler, Beckwith & Jackson, 1976). In the case of GSH, this thiol and its adducts seem more
94 interesting since the enzymatic formation of GSH adducts is one of the main pathways for
95 xenobiotic detoxification in the cells. These GSH adducts are excreted by the cell, being less
96 toxic than the parental xenobiotic compounds. In addition, the high hydrophilic character of
97 the glutathionylated moiety of GSH adducts could avoid the diffusion of these molecules into
98 the cell (Sheehan et al., 2001).

99 Fliege and Metzler (2000) and Schebb et al. (2009) have investigated the reaction of GSH
100 and patulin in a buffer solution at pH 7.4, identifying numerous adducts containing 1, 2 and 3
101 molecules of GSH. However, the published studies carried out at the acidic pH of juices have
102 shown the degradation of patulin in presence of thiol compounds, after applying different
103 treatments, but none of them have so far demonstrated the formation of these thiol-patulin

adducts. Merkulow et al. (2002) evaluated the patulin degradation in presence of cysteine in synthetic media (pH=4) by using different conditions of temperature (from 4 to 40°C) and hydrostatic pressure (up 500 MPa), obtaining the best degradations under high temperature and pressure conditions. On the other hand, Reiss (1976) reported high degradation rates for patulin in water solution at pH 5 in presence of GSH after 8 days. However, Folger (2014) observed significant patulin degradation (≈20%) at pH 3.7 only when the mixture was boiled. Although the formation of the thiol-patulin adducts was not verified, these results also seem to indicate that patulin degradation in presence of thiols, at the usual acid pH of the apple juices (around 3.5), requires the activation of the reaction. In this sense, the use of novel non-thermal food processing techniques could be an option for the activation of chemical reactions. In line with this, Brow, Dai, Park, Wright, Gillman and Manderville (2002) obtained GSH-Ochratoxin A (OTA) adducts by irradiation of a water solution with 350 nm wavelength light. Accordingly, novel food processing techniques based on wide spectrum light treatments could be suitable for the activation of chemical reactions in food matrices that allows obtaining specific degradation products through a more controlled and safe xenobiotic degradation mechanism.

Therefore, the aim of this work was the patulin detoxification of apple juice by the formation of specific degradation compounds, the GSH-patulin adducts, induced by the application of pulsed light treatments. With that purpose, patulin degradation has been studied considering the operational parameters of pulsed light treatments and the composition of the juice. In addition, the type of GSH-patulin adducts formed has been tentatively identified.

2. Materials and methods.

2.1. Reagents.

129 Patulin pure standard and dibasic potassium phosphate trihydrated were purchased from
130 Sigma-Aldrich (Sant Louis, MO, USA). Sodium hydroxide and hydrochloric acid solutions,
131 sodium carbonate, anhydrous sodium sulphate and citric acid from Panreac (Barcelona, Spain).
132 Formic acid, malic acid and ferrous sulphate heptahydrate from VWR (Fontenay-sous-Bois,
133 France). Acetic acid, methanol HPLC grade, ethyl acetate and sucrose from Fisher Scientific
134 (Loughborough, UK). Tetrahydrofurane (THF) from Scharlau (Barcelona, Spain) and GSH from
135 Across Chemicals (Geel, Belgium). Milli-Q water was obtained from a Millipore system
136 (Milford, USA).
137
138 2.2. Preparation of model solutions and apple juices.
139 Model solutions were prepared in a McIlvaine buffer (0.1 M solution of citric acid and 0.2 M
140 solution of potassium phosphate (7:3)), with and without sucrose (100g/L), and adjusted to pH
141 3.5 with a sodium hydroxide solution. A commercial filtered and pasteurized apple juice (105
142 g/L of sugars and pH=3.51) purchased in retail stores with no detectable patulin level was used
143 as juice standard reference. In addition, a model juice solution was prepared by dissolving of 5
144 g/L of L-malic acid and 100 g/L of sucrose and adjusted with hydrochloric acid to pH 3.5.
145 Samples were spiked with 83 µg/L of patulin (a concentration slightly higher than the regulated
146 limit) by using a concentrated standard solution of patulin (125 mg/L). GSH (50 g/L) and
147 ferrous sulfate heptahydrate (10 g/L) was dissolved in water and acidified water, respectively,
148 and the samples were fortified according to each experiment. Petri dishes were used as
149 sample container for the pulsed light treatments. All experiments were carried out in
150 duplicate.
151
152 2.3. Pulsed light processing.
153 Pulsed light treatments were applied using a lab-scale system (Steribeam Xe-Matic-2L-A,
154 Kehl, Germany) equipped with 2 lamps (quartz Xenon- gas pressure 450 Torr) situated above

and below the position of the sample with a spectrum range from 1100 to 180 nm. For establishing the energy doses supplied to the samples, a photodiode detector placed at the sample holder and connected to an oscilloscope was used. A calibration source equipped with a standard light according to the manufacturer instructions was also used (Maftei, Ramos-Villarroel, Nicolau, Martín-Belloso & Soliva-Fortuny, 2014; Avalos-Llano, Martín-Belloso & Soliva-Fortuny, 2018). The fluence was 0.4 J/cm^2 per pulse and lamp. The number of pulses applied to the samples ranged from 10 to 80 (from 8 to 64 J/cm^2) depending on the experiment. In addition, due to the limited transparency of the juices, the thickness of the liquid phase (in the Petri dish) was also considered. Both operating parameters (number of pulses and the thickness of the liquid phase) were optimized considering the usual working range found in the literature for similar purposes (Funes et al., 2013; Maftei et al., 2014).

2.4. Optimization of treatment conditions.

For the optimization of the treatment parameters (GSH and Fe^{2+} concentrations, number of light pulses (PL), and the thickness of the liquid phase (TL)), a 3-level-4-factor central composite design (CCD) with face centered axial points (α=1) and three replicates of the center point was designed. The conditions for the 27 runs of the CCD and the results for patulin content reduction (expressed in %) were presented in **Table 1**. Experiments were carried out with a commercial apple juice fortified with patulin (83 mg/L), GSH (50-125-250 mg/L) and Fe^{2+} (0-2-10 mg/L). The TL in the Petri dish was 0.33, 0.67 and 1 cm (for 20, 40 and 60 mL of liquid sample, respectively). The number of light pulses was 10, 20 and 30, equivalent to 8, 16 and 24 J/cm^2. Response surface methodology was used to model and optimize patulin content reduction (%) according with **equation 1**, where $\hat{Y}$ is the value estimated with the model, and $b_{i,j}$ the regression coefficients (0 is the intercept, 1 is GSH, 2 is Fe, 3 is TL, and 4 is PL) that includes the lineal and quadratic effects and the two way interactions. Non-significant effects ($p > 0.05$) were not considered in order to obtain a more accurate model. Results of the

ANOVA and regression coefficients are presented in Tables S1 and S2 (in supplementary information), and the residuals analysis (including Durbin-Watson test) in Figure S1 (in supplementary information). Predicted values are presented in **Table 1.**

$$\hat{Y} = b_0 + b_1 \cdot GSH + b_2 \cdot Fe + b_3 \cdot TL + b_4 \cdot PL + b_{1,1} \cdot GSH^2 + b_{2,2} \cdot Fe^2\ b_{3,3} \cdot TL^2 + b_{4,4} \cdot PL^2 + b_{1,2} \cdot GSH \cdot Fe + b_{1,3} \cdot GSH \cdot TL + b_{1,4} \cdot GSH \cdot PL + b_{2,3} \cdot Fe \cdot TL + b_{2,4} \cdot Fe \cdot PL + b_{3,4} \cdot TL \cdot PL \quad (\textbf{Eq. 1})$$

2.5. Patulin analysis by HPLC-DAD.

Patulin analysis of juice samples was carried out according to AOAC method 995.10 (Brause, Trucksess, Thomas & Page, 1996). Namely, 5 mL of sample were extracted twice with 5 mL of ethyl acetate. The extract was washed with 2 mL of sodium carbonate solution (1.5% w/w) and dehydrated with anhydrous sodium sulphate. After evaporation under nitrogen at 40°C, the residue was resuspended in 0.5 mL of acetic acid solution at pH 4 and was filtrated through 0.22 µm. Fifty µL were injected in the chromatographic system.

Chromatographic analysis was carried out by using an Agilent 1260 HPLC system (Wilmington, DE, USA) equipped with an autosampler and a diode array detector (DAD). The column was a reversed-phase C18 (150 mm x 4.6mm, 5µm of particle size) from Phenomenex (Torrance, USA). The mobile phase was Water-THF (0.8%) at a flow of 1 ml/min. The detection wavelength was 276 nm. Quantification was carried out by using patulin calibration curves prepared in synthetic juice and extracted according to the analytical method.

2.6. Tentative identification of GSH-Patulin adducts.

Only for identification purposes, the working concentrations of patulin, GSH and Fe^{+2} were increased proportionally in the solutions in order to have enough sensitivity in the direct injections of the samples in the chromatographic system. For obtaining the GSH-patulin adducts described by Fliege et al. (2000), 3 mL of patulin solution (125 mg/L) were evaporated to dryness under nitrogen and the residue was re-suspended in 0.6 mL of 0.5 M phosphate

207 buffer solution fortified with 33 mM of GSH at pH 7.5 (final concentration of patulin 625 mg/L).
208 In same way, another sample was resuspended in a synthetic juice (5 g/L of malic acid)
209 fortified with 33 mM of GSH and 6.5 mM of Fe^{2+} (ratio molar GSH/Fe^{2+} ≈ 5) at pH 3.5 and was
210 treated with pulsed light (78 pulses) in order to induce the complete degradation of patulin
211 and the formation of the GSH-patulin adducts. Apple juice samples were fortified with 200
212 mg/L of patulin, 13 mM of GSH and 2.6 mM of Fe^{2+} and were treated with pulsed light. Samples
213 (dilutions 1:10, 1:100 and 1:1000) were injected in the liquid chromatographic (LC) system.
214 The LC system was a Waters Acquity UPLC equipped with a binary pump, an autosampler
215 and a heated column compartment (30°C). The column was an ACQUITY UPLC® BEH C18 1.7µm
216 (2.1x150 mm) from Waters. The mobile phases were A (water:methanol (98:2)-0.1% formic
217 acid)) and B (methanol). The system was operated with a flow of 0.4 ml/min and the gradient
218 applied was as follows: 0 min 98% A; t = 4 min 96% A; t = 8 min 85% A; t = 12 min 98% A.
219 Injection volume was 2.5 µL.
220 The detectors were a DAD and a Waters Acquity TQD tandem quadrupole mass
221 spectrometer. The electrospray ionisation (ESI) source was operated in positive mode. ESI
222 parameters were: desolvation temperature, 400°C; desolvation gas (N2) flow rate, 1000 L/h;
223 cone gas (N_2) flow rate, 150 L/h, capillary voltage, 3.5 kV; and source temperature, 150°C. The
224 acquisition data ranged from 400 to 800 m/z (MS1-scan mode and collision energy 5 eV). The
225 m/z for each GSH-patulin adduct was described by Schebb et al. (2009). According with these
226 authors, m/z=769 was the ion for GSH bi-substitutes C6 or C2 thiol-substituted thiolenolether
227 ketones; m/z=462 was the ion for mono-substituted dihydropyranones; and m/z=741 was the
228 ion for GSH bi-substitutes ketohexanoic acids. In addition, complete spectra (220-500 nm)
229 were obtained with a diode array detector.
230
231 2.7. Statistics.

One way-ANOVA and least significance difference (LSD) test were used to evaluate the effects of the different treatments and sample types. The optimization of the processing parameters was carried out by using an experimental design based on a central composite design, and analyzed by two way-ANOVA and surface response analysis. Statistical analyses were carried out by using STATISTICA program for Windows (version 7.1) (StatSoft, Inc., 2005, www.statsoft.com).

3. Results and Discussion

3.1. Effect of the composition of the media on patulin degradation.

Published studies focused on the degradation of patulin in juices do not generally allow establishing the main degradation mechanism, as degradation products have not been studied. In this study, a first set of assays were carried out in different media fortified with 83 µg/L patulin: a model solution (pH=3.5), a model solution with sucrose (100 g/L; pH 3.5), and a commercial apple juice. These solutions were used to evaluate the effect of the presence of GSH and the pulsed light treatments on patulin degradation. In addition, taking into account the possible effect of divalent metal ions in presence of reducing agents (Lee et al., 1987), the addition of Fe^{2+} was evaluated (10 mg/L), with and without the presence of GSH (125 mg/L).

Patulin degradation in untreated and treated samples with pulsed light (78 pulses, equivalent to 62.4 J/cm^2) are presented in **Table 2.** A slight effect on patulin degradation (lower than 15%) was observed in samples not subjected to pulsed light treatments (upper part of **Table 2**), either when Fe^{2+} or GSH were added separately. Interestingly, model solutions without sucrose supplemented with Fe^{2+} and GSH exhibited a strong patulin degradation of 72.1%. However, patulin degradation in sucrose solution and apple juice substantially decreased even after the addition of GSH and Fe^{2+}. These results suggest that the spontaneous patulin degradation observed in presence of GSH and Fe^{2+} is inhibited by sucrose and probably

by other components of the apple juice. In addition, this also suggests a likely catalytic role of divalent metal ions, as previously hypothesized by Lee et al. (1987).

The chemical mechanisms of patulin degradation in the acidic conditions of our experiments are not described in the literature. Fliege et al. (2000) studied the spontaneous reaction between GSH and patulin based on an initial nucleophilic addition of the thiol group of the GSH to one of the double bonds of patulin at the cell pH (7.4). The spontaneity of this reaction can be explained by the reactivity of thiolate ions, that are slightly present (<10%) at that pH (Wall, Oh, Diers & Landar, 2012). Under the acidic conditions of our study, the presence of the thiolate ions is practically negligible, hence the reaction does not take place. However, Fe^{2+} ions may probably act as a catalyst in the reaction between patulin and GSH. Hamed and Silver (1983) studied the behavior of ferrous ions in presence of GSH in aqueous acidic media. These authors observed that Fe^{2+} formed complexes with GSH bound by the carboxylated group. The activation of the reaction between patulin and GSH is probably due to an intramolecular proton transfer in the coordination sphere of the Fe-GSH complex from the thiol group (forming the thiolate anion) (Esterbauer, Zöllner, & Scholz 1975; Rojas-Cervellera, Raich, Akola & Rovira, 2017). However, the presence of other components of the matrix such as sugars could limit this activation.

Pulsed light treatments applied to apple juice and model solutions led to significantly higher patulin degradation rates compared to non-irradiated samples. Interestingly, pulsed light-treated apple juice without GSH or Fe^{2+} addition exhibited higher patulin degradation (≈ 50%) than its respective model solutions, probably due to the presence of naturally present compounds (mainly proteins and peptides) containing thiol groups. Similar degradation percentages were observed by Funes et al. (2012) in apple juices treated with pulsed light. On the other hand, pulsed light-treated model solutions and apple juices supplemented with both, Fe^{2+} and GSH showed total or almost total patulin degradation. In the case of GSH-supplemented model solutions, sucrose apparently acted as an inhibitor of the degradation, as

observed in untreated solutions. However, patulin degradation in apple juice was close to 90%, corroborating again that other components of the food matrix could enhance the detoxification process. For a supplementation only with Fe^{2+}, the degradation percentage observed was higher than 79%, in the case of apple juice, and higher than 96.8%, for both model solutions.

These results show the importance of the combination of GSH and Fe^{2+} for an effective reduction of patulin levels by using pulsed light treatments. Thus, the individual effect of Fe^{2+} and GSH were clearly enhanced by the application of pulsed light, which suggests that photochemical activation of the reaction was probably involved (Brow et al., 2012). This photoactivation could be related to an intramolecular proton transference process in the complex Fe^{2+}-GSH, which has been reported to enhance the global reaction (Görner, Khanra, Weyhermüller, & Chaudhuri, 2006). The effectiveness of pulsed light could be though limited by the presence of other compounds in the treated matrix. On the other hand, pulsed light treatment produced lower patulin degradation for non supplemented model solutions than those observed by Funes et al. (2013). When Fe^{2+} ions were added, a strong enhancement of patulin degradation is achieved; however, in this case, the role of ferrous ions is not clear.

A study carried out by Palgan et al. (2011) showed that pulsed light treatments can affect the organoleptic characteristics of apple juices when high energy doses were applied. In order to evaluate the effectiveness of the treatment at lower energy doses than that applied in the initial experiments, a study of the degradation kinetics was carried out. To this purpose a apple juice samples supplemented with GSH (125 mg/L) and Fe^{2+} (10 mg/L) were treated with pulsed light at different energy doses (from 8 to 64 J/cm^2) (**Figure 1**). The percentage of degraded patulin increased rapidly when increasing the treatment intensity. As a treatment delivering an overall fluence of 24 J/cm^2 led to a 74% reduction of patulin content, this was established as the maximum energy for subsequent optimizations.

309 3.2. Optimization of the treatment for patulin degradation.

310 The effects of the addition of GSH, addition of Fe^{2+}, thickness of the liquid phase (TL) and
311 number of pulses (PL) on patulin degradation were evaluated by using a CCD. The analysis of
312 the experimental design (linear, quadratic and 2-way interaction effects according to **equation**
313 **1**) revealed significant effects (ANOVA, $p<0.05$) for GSH (linear and quadratic), PL (linear), Fe^{2+}
314 (linear), TL (linear) and 2-way interactions between GSH and Fe^{2+}, Fe^{2+} and PL, and PL and TL. A
315 Pareto chart showing significant effects is provided in **Figure 2**. As can be seen, Fe^{2+} and
316 number of light pulses, Fe^{2+}-PL interaction and TL were the factors with the strongest effects.
317 As expected, the thickness of liquid phase had an important negative effect. Furthermore, the
318 negative interactive effect of TL and PL shows the relationship between these two parameters
319 in terms of energy dose per amount of sample. Therefore, the juice transparency is an
320 important factor limiting the effectiveness of the light treatment (Oms-Oliu, Martín-Belloso &
321 Soliva-Fortuny, 2011). On the other hand, it is remarkable that the linear and quadratic effects
322 of GSH content were less intense than the interaction between GSH and Fe^{2+}, showing again
323 the importance of the combined action of both components.

324 With regard to the influence of process parameters, namely PL and TL, a response surface
325 analysis for the central values of GSH (125 mg/L) and Fe^{2+} (2 mg/L) shows the highest patulin
326 degradation for the highest dose (30 pulses) and the lowest TL (0.33 mm) (**Figure 3a**). These
327 conditions are equivalent to an energy dose of 24 J/cm^2 per 20 ml of juice, which is lower than
328 the doses applied by other authors without affecting the organoleptic quality of the juice
329 (Palgan at al., 2011). Regarding the effect of the addition of GSH and Fe^{2+}, the response surface
330 analysis for constant PL (30 pulses) and TL values (0.33 cm) (**Figure 3b**) clearly shows the
331 quadratic dependence of patulin degradation as a function of the GSH content. Hence, when
332 Fe^{2+} was not added, a maximum degradation was observed around 150 mg/L of added GSH.
333 However, the addition of Fe^{2+} produced a steep linear positive effect that compensated the

quadratic negative effect of GSH. These results indicate again that the addition of Fe^{2+} could enhance the effect of the GSH, hence, sustaining the idea of a catalytic role of Fe^{2+}.

3.3. Evaluation of the effect of the molar ratio GSH/Fe^{2+}.

In order to more deeply evaluate the role of $Fe^{2+}$and the interaction between Fe^{2+} and GSH, patulin degradation with different GSH/Fe^{2+} molar ratios was studied in synthetic media for a fixed GSH level of 150 mg/L and optimal operational conditions as defined in section 3.2 (0.33 mm of TL and 30 PL). The results are shown in **Table 3**. As can be seen, molar ratios from 0.5 to 10 allowed patulin degradations greater than 90% with a maximum value for a ratio of 5 (150 mg/L of GSH and 5.5 mg/L of Fe^{2+}). For very high ratio values (> 10), the effectiveness of the degradation process abruptly decreased.

According to Hamed et al. (1983), the stoichiometry of the complex Fe^{2+}-GSH formed is 1:1. However, taking into account that this complex is generated through the carboxylated group of the GSH, and considering the ionization equilibrium of GSH, only a part of the added GSH is available to form the complex with Fe^{2+}, which explains the high patulin degradation rates observed at GHS/Fe^{2+} ratios of 5 or 10. For ratios higher than 10 probably not all ionized GSH is complexed, which produces a reduction of the effectiveness of the light dose applied.

3.4. Tentative identification of degradation products: GSH-Patulin conjugates.

The published studies about the formation and identification of GSH-patulin adducts have been carried out in synthetic media under slightly basic conditions (Fliege et al. 2000; Schebb et al. 2009). However, in acidic conditions, such as those of an apple juice, literature does not provide evidence of the formation of these adducts. In order to evaluate if the same type of adducts than those described in bibliography is generated under acidic media, model solutions (at pH 3.5) and apple juices were treated with pulsed light and were compared with a reference solution at pH 7.5 obtained according to Fliege et al. (2000). Samples were injected

360 in a UPLC system coupled to a MS and DAD detector, and the m/z ions characteristic of the
361 known adducts were evaluated and compared.

362 **Figure S2** (supplementary information) shows the chromatograms of the samples at pH 7.5
363 (dilution 1/100) and 3.5 (dilution 1/100 and 1/1000) for the m/z ions 462, 741 and 769, and the
364 DAD and/or MS spectra of the identified compounds. As can be seen, the major peak for m/z
365 741 was detected at 5.05 min for solution at pH 7.5 (**Figure S2a**). According to Schebb et al.
366 (2009), this peak could correspond to bi-substituted ketohexanoic acid (compound type A in
367 figure S1a). For m/z 769 (**Figure 2Sb**), the solution at pH 7.5 also presented the biggest peak at
368 4.05 min. According to Schebb et al. (2009), this peak is likely a C6 or C2 thiol-substituted
369 thiolenolether ketone, which is a bi-substituted GSH-patulin adduct. Its UV spectrum, which
370 peaks around 305 nm, allows identifying it as the compound type B in **figure S2b** (according to
371 Fliege et al. (2000)). For m/z 462, the biggest peaks were observed under acidic conditions at
372 7.15 and 7.25 min, being identified as mono-substituted dihydropyranones (type C compound
373 in **figure S2c**) (Schebb et al., 2009). In addition, the UV spectra of both peaks showed a
374 maximum at 272 nm, in accordance with the results of Fliege et al. (2000). These two peaks
375 corresponding to mono-subtituted adduct forms were not detected in the samples at pH 7.5.
376 In a similar way, the bi-substituted GSH adducts were not detected or were present in an
377 almost negligible amount in the samples at pH 3.5 compared to the mono-substituted
378 compounds.

379 These results suggest that the formation of more simple adducts, with only one GSH
380 substituted group is favored under acidic pH conditions. However, the formation of adducts
381 with multiple GSH groups is more common at pH 7.5. As outlined in previous sections, the
382 reactivity of GSH, due to the presence of thiolate ion, is much higher at basic pH (Wall et al.,
383 2012), which could explain the type of adducts obtained depending on the pH. Moreover, the
384 reaction at acidic pH requires the formation of a complex that could limit the multiple
385 additions of GSH groups due to steric hindrance.

386 **Figure S3** (supplementary information) shows the chromatograms (m/z 462, 741 and 769)
387 for a treated apple juice. As expected, the profile was similar to the obtained in the model
388 solution at pH 3.5, clearly showing the two peaks of the GSH-patulin mono-substituted forms
389 at 7.15 and 7.25 min. Therefore, mono-substituted dihydropyranones would be the main
390 adduct obtained in real juices after the treatment. Pfenning, Esch, Fliege & Lehmann (2016)
391 observed that these mono-GSH-substituted patulin adducts retained the capacity to bind to
392 the DNA nucleosides (adenine, guanine, thymine and cytosine). Although these authors carried
393 out *in vitro* experiments in presence of GSH, nucleosides and the enzyme glutathione S-
394 transferase in order to emulate the cell media, the diffusion of these molecules into the cell is
395 not likely to occur taking into account its structure and its hydrophilic character (Sheehan et
396 al., 2001).
397 Therefore, the procedure presented in this study allows an effective degradation of patulin
398 to GSH-patulin adducts, being these products less toxic than the parental mycotoxin (Sheehan
399 et al., 2001). Compared to the previously published studies addressing the patulin degradation
400 in apple-derived products, the main innovation of this study is the use of non-thermal
401 processing to promote the formation of specific degradation products.
402 **Conclusions**
403 A novel strategy for patulin degradation in apple juices is proposed. After supplementation
404 of the juices with GSH, the formation of the GSH-patulin adducts was induced by means of the
405 application of pulsed light treatment. In addition, the presence of ferrous ions enhanced
406 patulin degradation, these ions being a catalyst of the chemical reaction between patulin and
407 GSH. The identification of the degradation products in apple juices indicated that the main
408 adducts formed were the GSH mono-substituted adducts. More research is necessary in order
409 to evaluate the behavior of these adducts in the body after ingestion. As well, further studies
410 are required to optimize processing parameters with regard to oxidative stability and sensory
411 quality of the juices.

Acknowledgments

J. J R.- B. appreciates the postdoctoral contract from Agrotecnio center.

Supplementary information description

Table S1: Analysis of variance (ANOVA) of the central composite design for patulin content reduction.

Table S2: Regression coefficient results from the data of central composite design experiments for patulin content reduction.

Figure S1: Analysis of residuals of the response surface analysis of the central composite design.

Figure S2: Ion chromatograms (m/z=741 (fig. S2a); m/z=769 (fig. S2b); and m/z=462 (fig. S2c) for solutions at pH 3.5 and 7.5 containing GSH-patulin adducts. UV and/or MS spectra of the compounds tentatively identified. Compound structures were obtained from Fliege et al. (2000) and Schebb et al. (2009). GS indicates glutathionyl substitutions.

Figure S3: Ion chromatograms of m/z 741, 769 and 462 for pulsed light-treated apple juice fortified with glutathione, patulin and ferrous ion.

428 **References**

429 Acar, J., Gokmen, V., and Taydas, E. E. (1998). The effects of processing technology on the
430 patulin content of juice during commercial apple juice concentrate production. *Zeitschrift*
431 *für Lebensmittel-Untersuchung und -Forschung A, 207*, 328–331. doi:
432 10.1007/s002170050341

433 Avalos-Llano, K. R.; Martín-Belloso, O., and Soliva-Fortuny, R. (2018). Effect of pulsed light
434 treatments on quality and antioxidant properties of fresh-cut strawberries. *Food Chemistry,*
435 *264,* 393-400. doi: 10.1016/j.foodchem.2018.05.028

436 Avsaroglu, M. D., Bozoglu, F., Alpas, H., Largeteau, A., and Demazeau, G. (2015). Use of pulsed-
437 high hydrostatic pressure treatment to decrease patulin in apple juice. *High Pressure.*
438 *Research, 35*, 214–222. doi: 10.1080/08957959.2015.1027700

439 Bissessur, J., Permaul, K., and Odhav, B. (2001). Reduction of patulin during apple juice
440 clarification. *Journal of Food Protection, 64*, 1216–1219. doi: 10.4315/0362-028X-64.8.1216

441 Brause, A. R., Trucksess, M. W., Thomas, F., and Page, S. W. (1996). Determination of patulin in
442 apple juice by liquid chromatography: collaborative study. *Journal of AOAC International,*
443 *79,* 451-455.

444 Brow, M. E., Dai, J., Park, G., Wright, M. W., Gillman, I. G., and Manderville, R. A. (2002).
445 Photochemically catalyzed reaction of ochratoxin A with D – and L –cysteine.
446 *Photochemistry and Photobiology, 76,* 649–656. doi: 10.1562/0031-
447 8655(2002)076<0649:PCROOA>2.0.CO;2

448 CE No 1881/2006. Diario Oficial de la Unión Europea de 19 de diciembre de 2006.

449 Ciegler, A., Beckwith, A. C., and Jackson, L. K. (1976). Teratogenicity of patulin and patulin
450 adducts formed with cysteine. *Applied and Environmental Microbiology, 31*, 664–667.

451 Drusch, S., Kopka, S., and Kaeding, J. (2007). Stability of patulin in a juice-like aqueous model
452 system in the presence of ascorbic acid. *Food Chemistry, 100,* 192–197. Doi:
453 10.1016/j.foodchem.2005.09.043

454 Esterbauer, H., Zöllner, H., and Scholzn N. (1975). Reaction of glutathione with conjugated
455 carbonyls. *Zeitschrift für Naturforschung C, 30*, 466-473. doi: 10.1515/znc-1975-7-808
456 Fliege, R., and Metzler, M. (2000). Electrophilic properties of patulin. N-acetylcysteine and
457 glutathione adducts. *Chemical Research in Toxicology, 13*, 373-381. doi: 10.1021/tx9901480
458 Folger, B.C. (2014). Patulin degradation by yeast protein extract. Master Thesis. University of
459 Minnesota.
460 Funes, G. J., Gómez, P. L., Resnik, S. L., and Alzamora, S. M. (2013). Application of pulsed light
461 to patulin reduction in McIlvaine buffer and apple products. *Food Control, 30*, 405-410. doi:
462 10.1016/j.foodcont.2012.09.001
463 Gökmen, V., Artık, N., Acar, J., Kahraman, N., and Poyrazoglu, E. (2001). Effects of various
464 clarification treatments on patulin, phenolic compound and organic acid compositions of
465 apple juice. *European Food Research and Technology, 213*, 194–199. doi:
466 10.1007/s002170100354.
467 Görner, H., Khanra, S., Weyhermüller, T., and Chaudhuri, P. (2006). Photoinduced
468 intramolecular proton transfer of phenol-containing ligands and their zinc complexes. *The*
469 *Journal of Physical Chemistry A, 110*, 2587-2594. doi: 10.1021/jp053662p
470 Hamed, M. Y., and Silver, J. (1983). Studies on the reactions of ferric iron with glutathione and
471 some related thiols. Part II. Complex formation in the pH range three to seven. *Inorganica*
472 *Chimica Acta, 80*, 115-122. doi: 10.1016/S0020-1693(00)91271-4
473 Hao, H., Zhou, T., Koutchma, T., Wu, F., and Warriner, K. (2016). High hydrostatic pressure
474 assisted degradation of patulin in fruit and vegetable juice blends. *Food Control, 62*, 237–
475 242. doi: 10.1016/j.foodcont.2015.10.042
476 Ioi, J. D., Zhou, T., Tsao, R., and Marcone, M. F. (2017). Mitigation of Patulin in fresh and
477 processed foods and beverages. *Toxins, 9*, 157. doi: 10.3390/toxins9050157
478 Jones, D. P., Coates, R. J., Flagg, E. W., Eley, J. W., Block, G., Greenberg, R. S., Gunter, E. W., and
479 Jackson, B. (1992). Glutathione in foods listed in the national cancer institute's health habits

480 and history food frequency questionnaire. *Nutrition and Cancer, 17*, 57-75. doi:
481 10.1080/01635589209514173
482 Kadakal, C., and Nas, S. (2003). Effect of heat treatment and evaporation on patulin and some
483 other properties of apple juice. *Journal of the Science of Food and Agriculture, 83*, 987–990.
484 doi: 10.1002/jsfa.1339
485 Lee, K. S., and Roschenthaler, R. (1987). Strand scissions of DNA by patulin in the presence of
486 reducing agents and cupric ions. *The Journal of Antibiotics, 40*, 692-696. doi:
487 10.7164/antibiotics.40.692
488 Lindroth, S., and Von Wright, A. (1978). Comparison of the toxicities of patulin and patulin
489 adducts formed with cysteine. *Applied and Environmental Microbiology, 35*, 1003-1007.
490 Maftei, N. A., Ramos-Villarroel, A. Y., Nicolau, A. I., Martín-Belloso, O., and Soliva-Fortuny, R.
491 (2014). Influence of processing parameters on the pulsed-light inactivation of *Penicillium*
492 *expansum* in apple juice. *Food Control, 41*, 27-31. doi: 10.1016/j.foodcont.2013.12.023
493 Merkulow, N., and Ludwig, H. (2002). The influence of high hydrostatic pressure on the adduct
494 formation of Patulin with Cysteine. In R. Hayashi (ed.), *Trends in High Pressure Bioscience*
495 *and Biotechnology* (pp. 349-354). Elsevier Science. doi: 10.1016/S0921-0423(02)80123-X
496 Oms-Oliu, G., Martín-Belloso, O., and Soliva-Fortuny, R. (2010). Pulsed light treatments for
497 food preservation. A review. *Food and Bioprocess Technology, 3*, 13-23. doi:
498 10.1007/s11947-008-0147-x
499 Pal, S., Singh, N., and Ansari, K. M. (2017). Toxicological effects of patulin mycotoxin on the
500 mammalian system: an overview. *Toxicological Research, 6*, 764-771. doi:
501 10.1039/c7tx00138j
502 Palgan, I., Caminiti, A., Muñoz, A., Noci, F., Whyte, P., Morgan, D. J., Cronin D. A., and Lyng, J.
503 G. (2011). Effectiveness of height intensity light pulses (hilp) treatments for the control of
504 *Escherichia coli* and *Listeria innocua* in apple juice, orange juice and milk. *Food*
505 *Microbiology, 28*, 14-20. doi: 10.1016/j.fm.2010.07.023

506 Pfenning, C., Esch, H. L., Fliege, R., and Lehmann, L. (2016). The mycotoxin patulin reacts with
507 DNA bases with and without previous conjugation to GSH: implication for related α,β-
508 unsaturated carbonyl compounds?. *Archives of Toxicology, 90*, 433-448. doi:
509 10.1007/s00204-014-1443-z
510 Puel, O., Galtier, P., and Oswald, I. P. (2010). Biosynthesis and toxicological effects of patulin.
511 *Toxins, 2*, 613-631. doi:10.3390/toxins2040613
512 Reiss, J. (1976). Mycotoxins in foodstuffs. VII. Inactivation of patulin in whole wheat bread by
513 sulfhydryl compounds. *Cereal Chemistry, 53*, 150-152.
514 Rojas-Cervellera, V., Raich, L., Akola, J., and Rovira, C. (2017). The molecular mechanism of the
515 ligand exchange reaction of an antibody against a glutathione-coated gold cluster.
516 *Nanoscale, 9*, 3121–3127. doi: 10.1039/c6nr08498b
517 Schebb, N. H., Faber, H., Maul, R., Heus, F., Kool, J., Irth, H., and Karst, U. (2009). Analysis of
518 glutathione adducts of patulin by means of liquid chromatography (HPLC) with biochemical
519 detection (BCD) and electrospray ionization tandem mass spectrometry (ESI-MS/MS).
520 *Analytical and Bioanalytical Chemistry, 394*,1361–1373. doi: 10.1007/s00216-009-2765-1
521 Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001). Structure, function and evolution
522 of glutathione transferases: implications for classification of non-mammalian members of
523 an ancient enzyme superfamily. *Biochemical Journal, 360*, 1-16. doi: 10.1042/0264-
524 6021:3600001
525 Wall, S. B., Oh, J. Y., Diers, A. R., and Landar, A. (2012). Oxidative modification of proteins: an
526 emerging mechanism of cell signaling. *Frontiers in Physiology, 14*, 369. doi:
527 10.3389/fphys.2012.00369.
528 Welke, J. E., Hoeltz, M., Dottori, H. A., and Noll, I. B. (2009). Effect of processing stages of apple
529 juice concentrate on patulin levels. *Food Control, 20*, 48–52. doi:
530 10.1016/j.foodcont.2008.02.001

Zhang, Z. H., Wang, L. H., Zeng, X. A., Han, Z., and Brennan, C. S. (2018). Non-thermal technologies and its current and future application in the food industry: a review. *International Journal of Food Science and Technology, 54*, 1-13. doi: 10.1111/ijfs.13903

Zhong, L., Carere, J., Lu, Z., Lu, F., and Zhou, T. (2018). Patulin in apples and apple-based food products: the burdens and the mitigation strategies. *Toxins, 10*, 475. doi:10.3390/toxins10110475.

Zhu, Y., Koutchma, T., Warriner, K., and Zhou, T. (2014). Reduction of patulin in apple juice products by UV light of different wavelengths in the UVC range. *Journal of Food Protection, 77*, 963–971. doi: 10.4315/0362-028X.JFP-13-429.

Figure captions

Figure 1. Kinetics of patulin content reduction in an apple juice fortified with 83 µg/L of patulin, supplemented with glutathione (125 mg/L) and Fe^{2+} (10 mg/L) and treated with pulsed light.

Figure 2. Pareto chart of the factors significantly affecting patulin degradation (Glutathione = GSH; Fe^{2+}; PL = number of light pulses and TL = thickness of the liquid phase). L: linear component. Q: quadratic component. Two way interactions are also indicated.

Figure 3. Surface plots of the estimated response (patulin content reduction (%)) based on the CCD design: a) Effect of light pulses and thickness of the liquid phase for glutathione (GSH) (125 mg/L) and Fe^{2+} (2 mg/L)). b) Effect of GSH and Fe^{2+} contents for 30 light pulses and 0.33 cm of thickness of liquid phase.

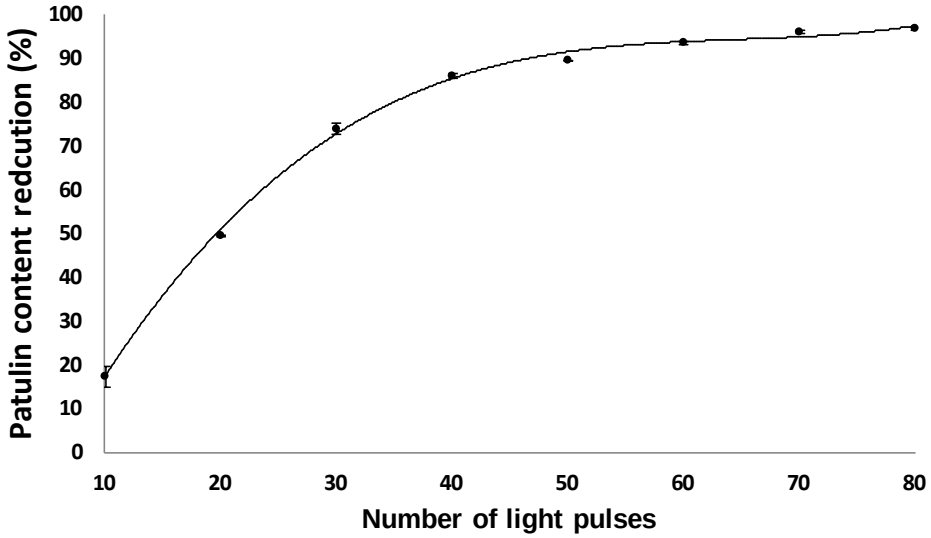
Patulin content redcution (%)
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Number of light pulses

Figure 1

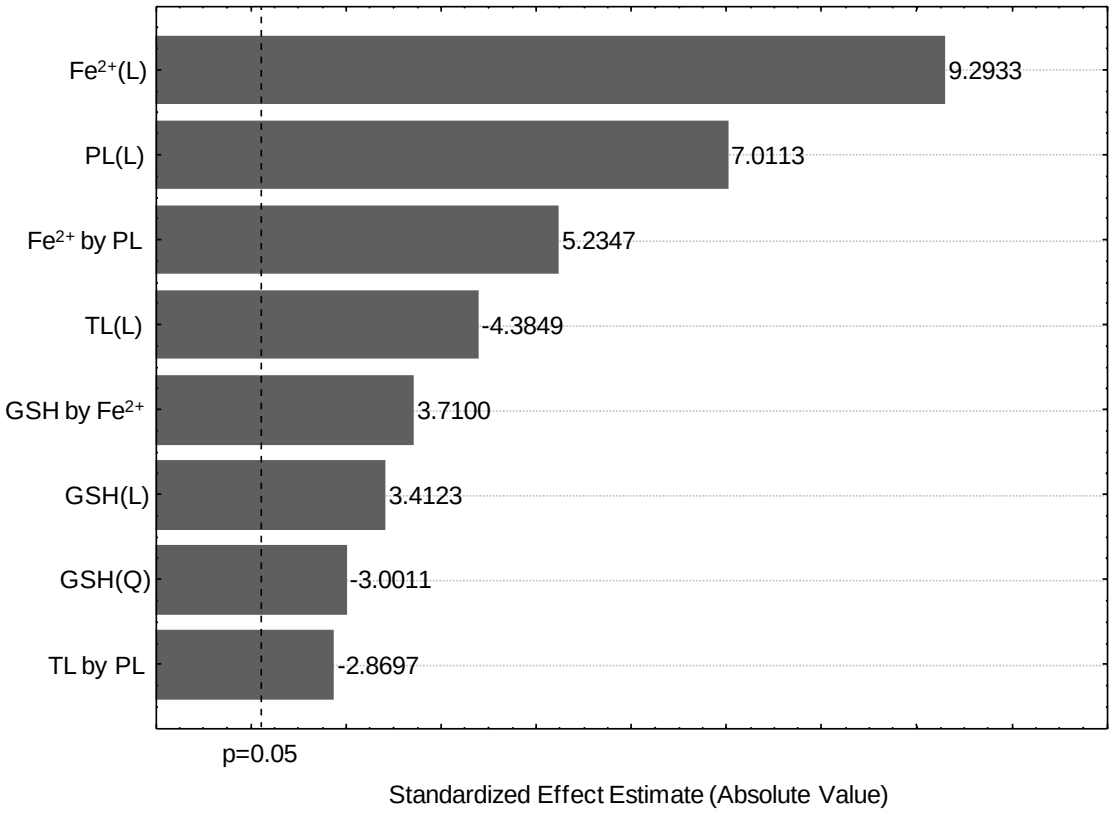
Fe2+(L)
PL(L)
Fe2+ by PL
TL(L)
GSH by Fe2+
GSH(L)
GSH(Q)
TL by PL
9.2933
7.0113
5.2347
-4.3849
3.7100
3.4123
-3.0011
-2.8697
p=0.05
Standardized Effect Estimate (Absolute Value)

Figure 2

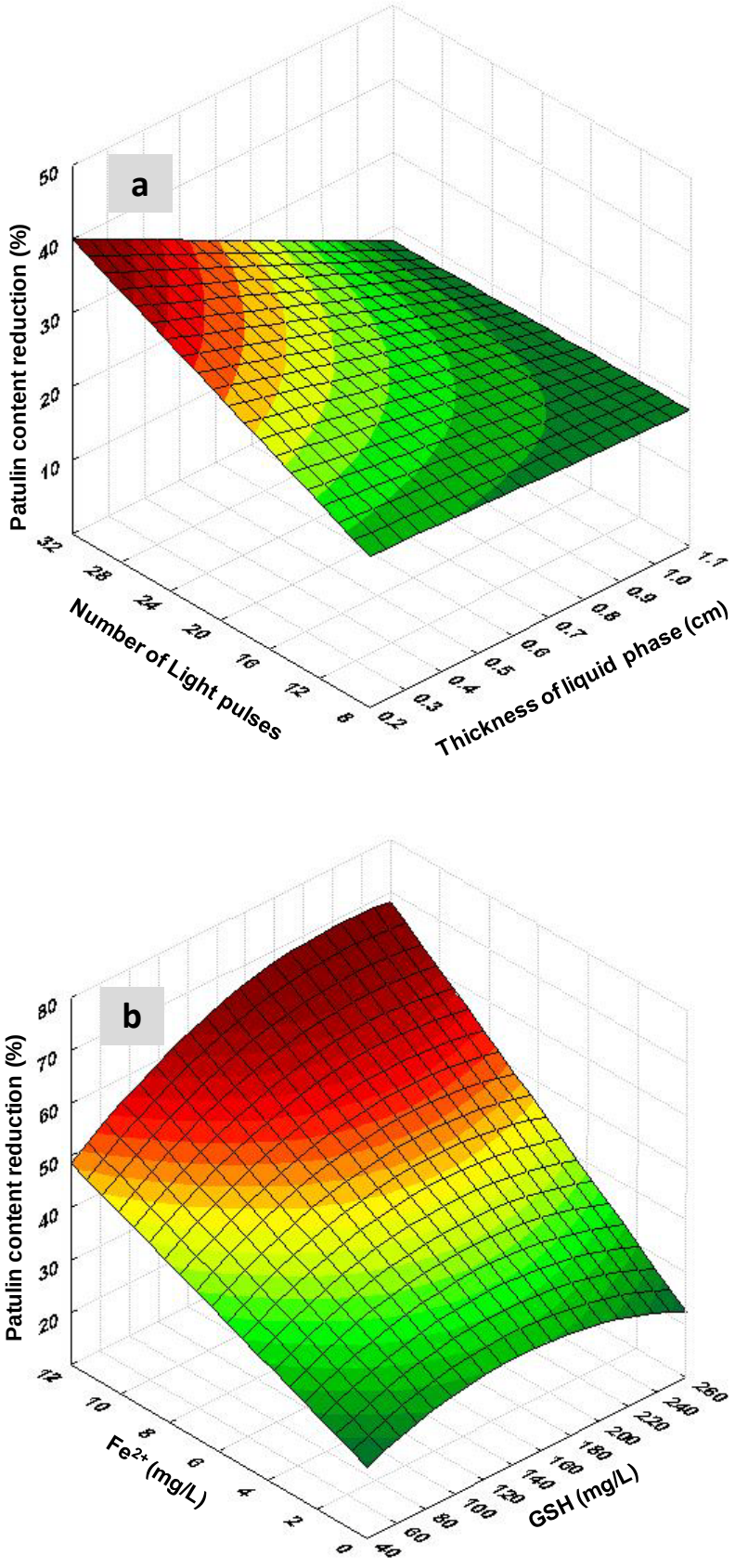
a
Patulin content reduction (%)
50
40
30
20
10
32
28
24
20
16
12
8
Number of Light pulses
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Thickness of liquid phase (cm)
b
Patulin content reduction (%)
80
70
60
50
40
30
20
12
10
8
6
4
2
0
Fe2+ (mg/L)
40
60
80
100
120
140
160
180
200
220
240
260
GSH (mg/L)

Figure 3

Table 1. Central composite design 3-level-4-factors with face centered axial points (α=1) and three replicates of the center point (C) for the optimization of the treatment for patulin degradation. The factors of the experimental design are: fortified glutathione (GSH); fortified Fe^{2+}; volume of sample in the Petri dish; and number light pulses applied. All samples were fortified with 83 µg/L of patulin.

Run	GSH (mg/L)	Fe^{2+} (mg/L)	Volume-(mL) (Thickness-(cm))	Number of Light Pulses	Observed Patulin Content Reduction (%)	Predicted[1] Patulin Content Reduction (%)
1	50	0	10 (0.33)	10	14.8	15.4
2	50	0	10 (0.33)	30	20.8	24.8
3	50	0	30 (1.0)	10	16.2	12.7
4	50	0	30 (1.0)	30	16.7	9.70
5	50	10	10 (0.33)	10	17.4	14.8
6	50	10	10 (0.33)	30	46.3	46.3
7	50	10	30 (1.0)	10	15.7	12.1
8	50	10	30 (1.0)	30	25.9	31.2
9	250	0	10 (0.33)	10	16.7	14.5
10	250	0	10 (0.33)	30	23.7	23.9
11	250	0	30 (1.0)	10	9.52	11.8
12	250	0	30 (1.0)	30	10.5	8.84
13	250	10	10 (0.33)	10	27.1	29.6
14	250	10	10 (0.33)	30	68.1	61.1
15	250	10	30 (1.0)	10	23.3	26.9
16	250	10	30 (1.0)	30	44.9	46.0
17	50	2	20 (0.66)	20	11.1	17.8
18	250	2	20 (0.66)	20	18.7	20.1
19	125	0	20 (0.66)	20	17.1	20.8
20	125	10	20 (0.66)	20	37.3	37.2
21	125	2	10 (0.33)	20	27.8	28.5
22	125	2	30 (1.0)	20	19.8	19.6
23	125	2	20 (0.66)	10	18.2	20.2
24	125	2	20 (0.66)	30	23.9	28.0
25 (C)	125	2	20 (0.66)	20	29.7	24.1
26 (C)	125	2	20 (0.66)	20	27.3	24.1
27 (C)	125	2	20 (0.66)	20	25.7	24.1

[1] Predicted values obtained from the fitted model.

Table 2. Patulin content reduction (%) [1] in the model solutions and apple juice [2] treated or not with pulsed light [3].

	Untreated with pulsed light [4]		
Sample type	***Solution pH 3.5***	***Sucrose solution pH 3.5***	***Apple juice***
GSH	11.8 ± 4.4 a,a	0.645±0.207 a,a	6.26 ± 1.17 a,a
Fe^{2+}	13.8 ± 3.9 a,b	1.06±2.88 a,a	13.6 ± 1.8 b,b
GSH + Fe^{2+}	72.1 ± 1.8 b,b	14.5 ± 6.24 b,a	8.69 ± 0.07 a,a
	Treated with pulsed light [4]		
Sample type	***Solution pH 3.5***	***Sucrose solution pH 3.5***	***Apple juice***
Control	21.1 ± 2.7 a,b	9.03 ± 1.29 a,a	50.7 ± 0.5 a,c
GSH	61.2 ± 6.1 b,b	29.1 ± 4.7 b,a	89.5 ± 2.1 c,c
Fe^{2+}	96.8 ± 0.4 c,b	100 c,b	79.0 ± 3.5 b,a
GSH + Fe^{2+}	100 c,b	100 c,b	97.3 ± 0.5 d,a

[1] All samples were fortified with 83 μg/L of patulin. Patulin content reduction percentages were calculated with respect to each untreated control sample (only fortified with patulin). [2] Solutions and juices were fortified with 100 g/L of sucrose, 125 mg/L of glutathione (GSH) and 10 mg/L of Fe^{2+}, depending on the type of sample. [3] Conditions of pulsed light treatment: 30 mL of sample and 78 light pulses (total energy dose: 62.4 J/cm^2). [4] First letter indicate statistical differences between sample type (vertical, $p<0.05$). Second letter indicate statistical differences between solution type (horizontal, $p<0.05$).

Table 3. Effect of the molar ratio Glutathione/Fe^{2+} on the patulin content reduction (%) [1] in model solutions.

Molar Ratio [2] (Glutathione/Fe^{2+})	Patulin Content Reduction (%) [3]
0.25	88.4 ± 0.5 c
0.5	94.0 ± 1.3 e
1	96.7 ± 0.5 f
5	98.5 ± 0.1 f
10	91.0 ± 0.3 d
25	62.4 ± 0.5 b
50	50.1 ± 2.1 a

[1] Patulin content reduction percentages were calculated respect to the untreated solution fortified only with patulin at 83 μg/L. Conditions of pulsed light treatment: 20 mL of sample (0.33 cm of thickness of liquid phase) and 30 light pulses (total energy dose: 24 J/cm^2). [2] For a fix glutathione dose of 150 mg/L. [3] Different letters indicate statistical differences ($p<0.05$).

Title: Formation of Patulin-Glutathione conjugates induced by pulsed light: a tentative strategy for patulin degradation in apple juices**.**

Authors: Juan José Rodríguez-Bencomo*; Vicente Sanchis; Inmaculada Viñas; Olga Martín-Belloso; Robert Soliva-Fortuny

Address: Food Technology Department, UTPV-XaRTA, Agrotecnio Center, University of Lleida, Rovira Roure 191, 25198, Lleida, Spain**.**

Running title: Patulin-Glutathione conjugates for degrading patulin in apple juices

*corresponding author: jrbencomo@gmail.com / pvrodriguez@tecal.udl.cat

Supplementary Information.

Table S1: Analysis of variance (ANOVA) of the central composite design for patulin content reduction.

Table S2: Regression coefficient results from the data of central composite design experiments for patulin content reduction.

Figure S1: Analysis of residuals of the response surface analysis of the central composite design.

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Figure S3: Ion chromatograms of m/z 741, 769 and 462 for pulsed light-treated apple juice fortified with glutathione, patulin and ferrous ion.

Table S1. Analysis of variance (ANOVA) of the central composite design for patulin content reduction.

	Sum of square	Degrees of freedom	Mean square	F-value	p-value
GSH (L)	216.044	1	216.044	11.64397	0.003106
GSH (Q)	167.110	1	167.110	9.00662	0.007667
Fe^{+2} (L)	1602.432	1	1602.432	86.36503	0.000000
TL (L)	356.743	1	356.743	19.22709	0.000357
PL (L)	912.098	1	912.098	49.15862	0.000002
GSH by Fe^{+2}	255.384	1	255.384	13.76422	0.001603
Fe^{+2} by PL	508.415	1	508.415	27.40163	0.000056
TL by PL	152.793	1	152.793	8.23498	0.010191
Error	333.975	18	18.554		
Total Sum. Square	4188.690	26			

Q: quadratic; L: Linear; - by –: linear interaction. Fe (Q), L (Q), Pulses (Q) and the interactions (GHS by TL; GSH by PL and Fe^{+2} by TL) were excluded due to the p value > 0.05. R^2= 0.92027; R^2-Adj=0.88483.

Table S2. Regression coefficient results from the data of central composite design experiments for patulin content reduction.

	Regression Coeff.	Std.Err.	t-value	p-value
Mean/Intercept.	1.90568	6.899463	0.27621	0.785536
GSH (L)	0.17069	0.061815	2.76136	0.012857
GSH (Q)	-0.00058	0.000194	-3.0011	0.007667
Fe^{+2} (L)	-1.55314	0.558757	-2.77964	0.012364
TL (L)	5.16012	7.107475	0.72601	0.477166
PL (L)	0.77423	0.256122	3.02289	0.007312
GSH by Fe^{+2}	0.0078	0.002103	3.71002	0.001603
Fe^{+2} by PL	0.11055	0.021119	5.23466	0.000056
TL by PL	-0.92245	0.321448	-2.86967	0.010191

Q: quadratic; L: linear; - by –: linear interaction. R^2= 0.92027; R^2-Adj=0.88483.

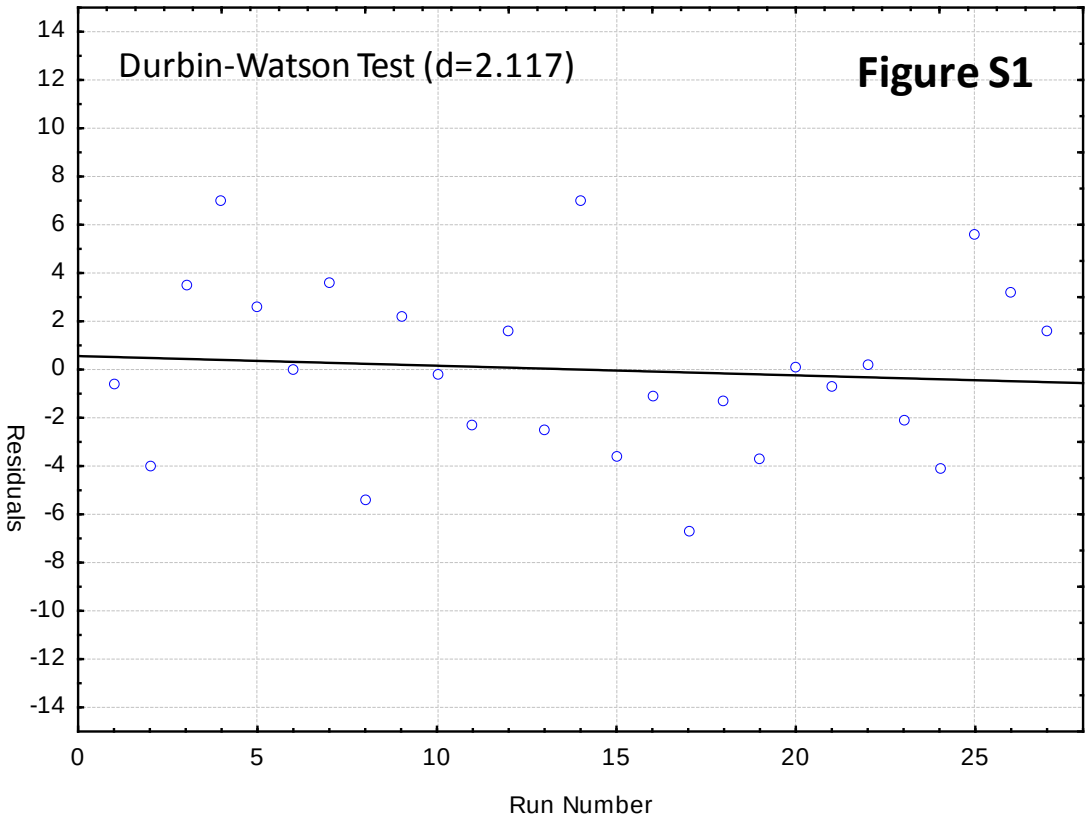
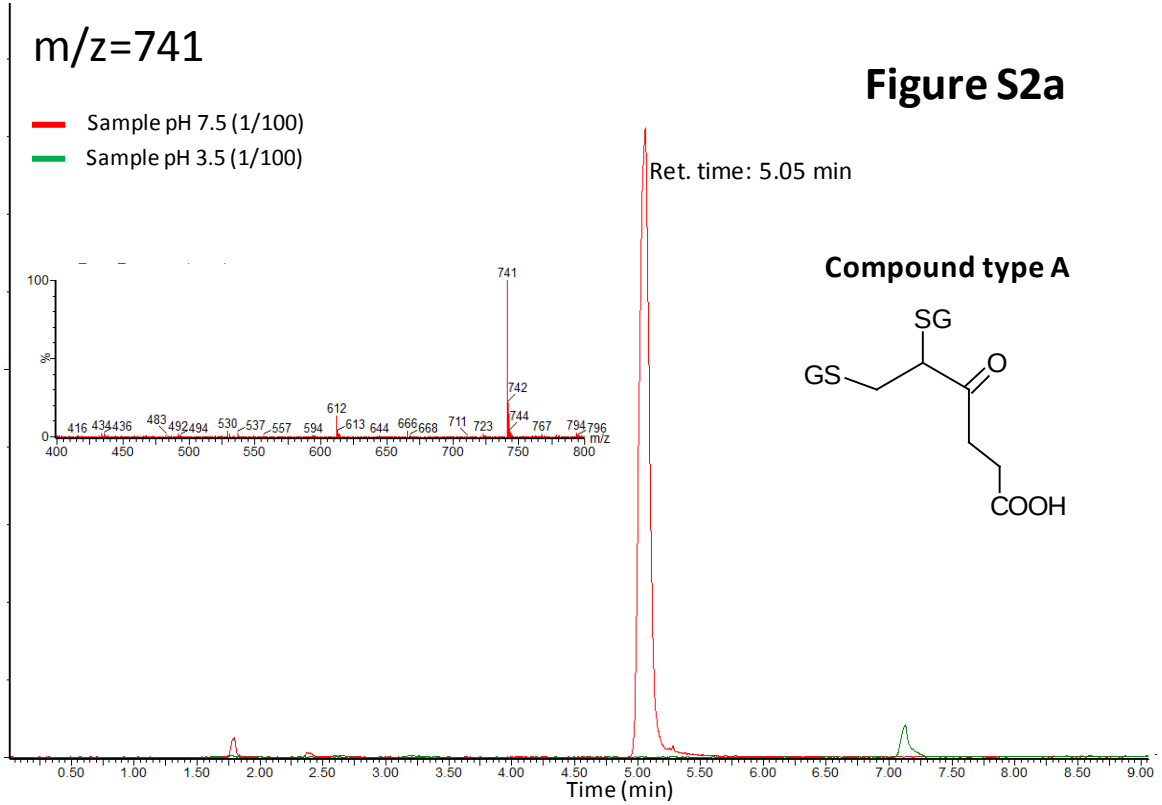

Durbin-Watson Test (d=2.117)
Figure S1
Residuals
Run Number
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
0
5
10
15
20
25

Figure S2a


m/z=741
Sample pH 7.5 (1/100)
Sample pH 3.5 (1/100)
Ret. time: 5.05 min
Compound type A
SG
GS
O
COOH
Time (min)

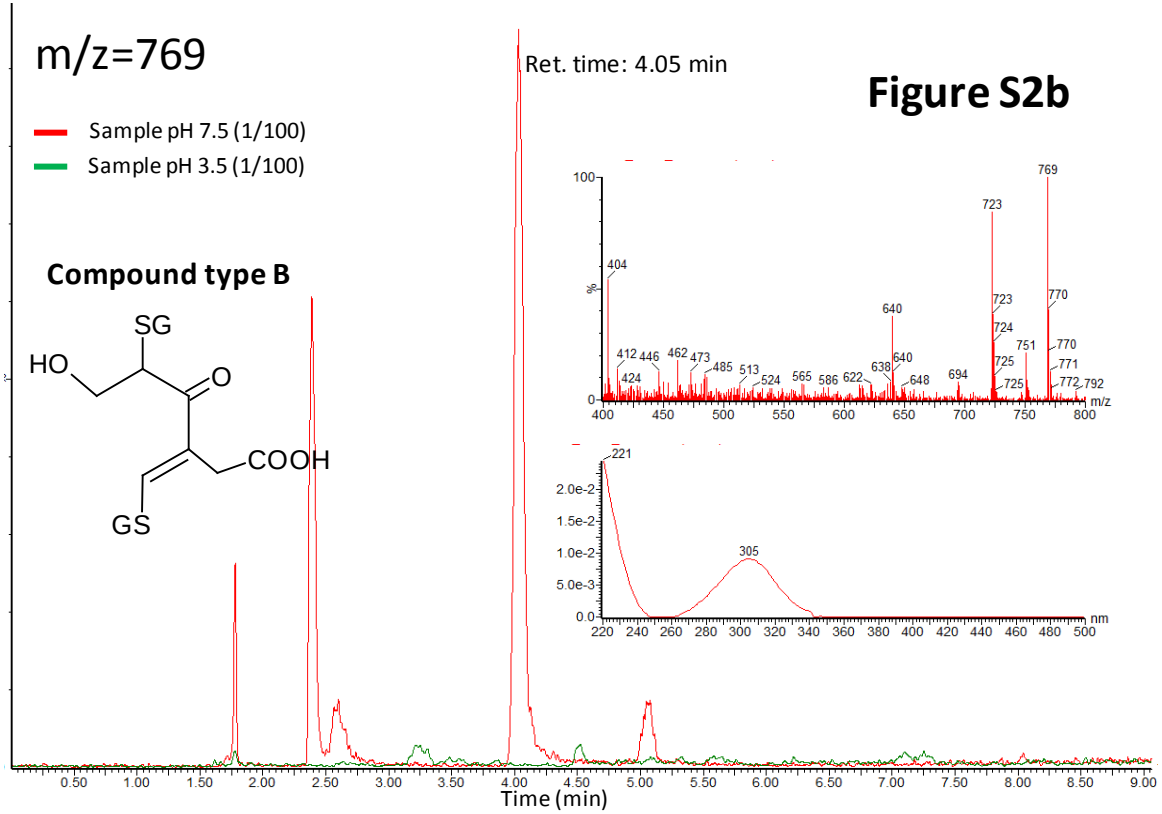

m/z=769
Sample pH 7.5 (1/100)
Sample pH 3.5 (1/100)
Ret. time: 4.05 min
Compound type B
SG
HO
O
COOH
GS
Time (min)
0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00 8.50 9.00
100
%
0
404
412 424 446 462 473 485 513 524 565 586 622 638 640 640 648 694 723 724 725 725 751 769 770 770 771 772 792
400 450 500 550 600 650 700 750 800
m/z
221
305
2.0e-2
1.5e-2
1.0e-2
5.0e-3
0.0
220 240 260 280 300 320 340 360 380 400 420 440 460 480 500
nm

Figure S2b

Figure S2c

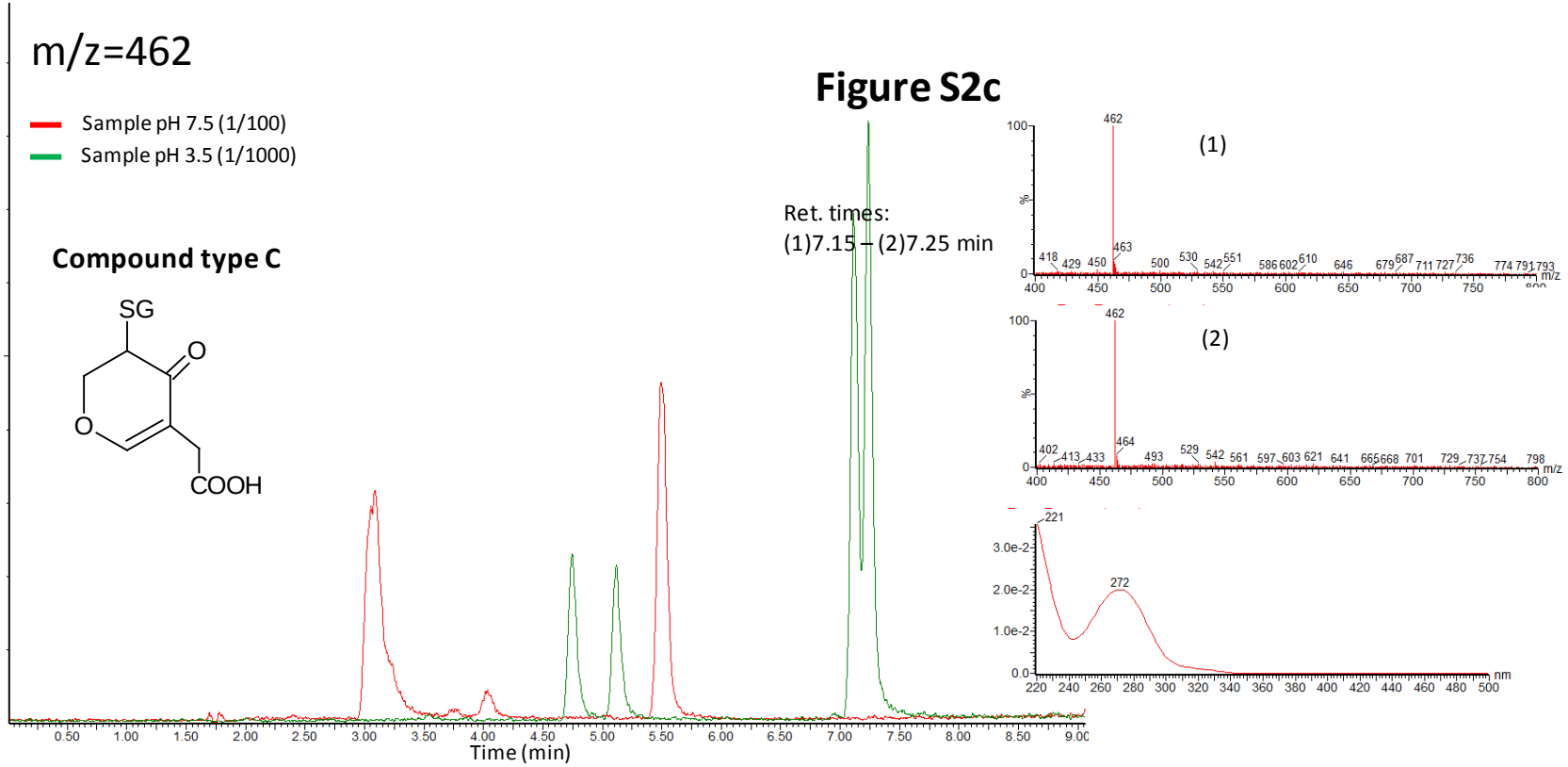
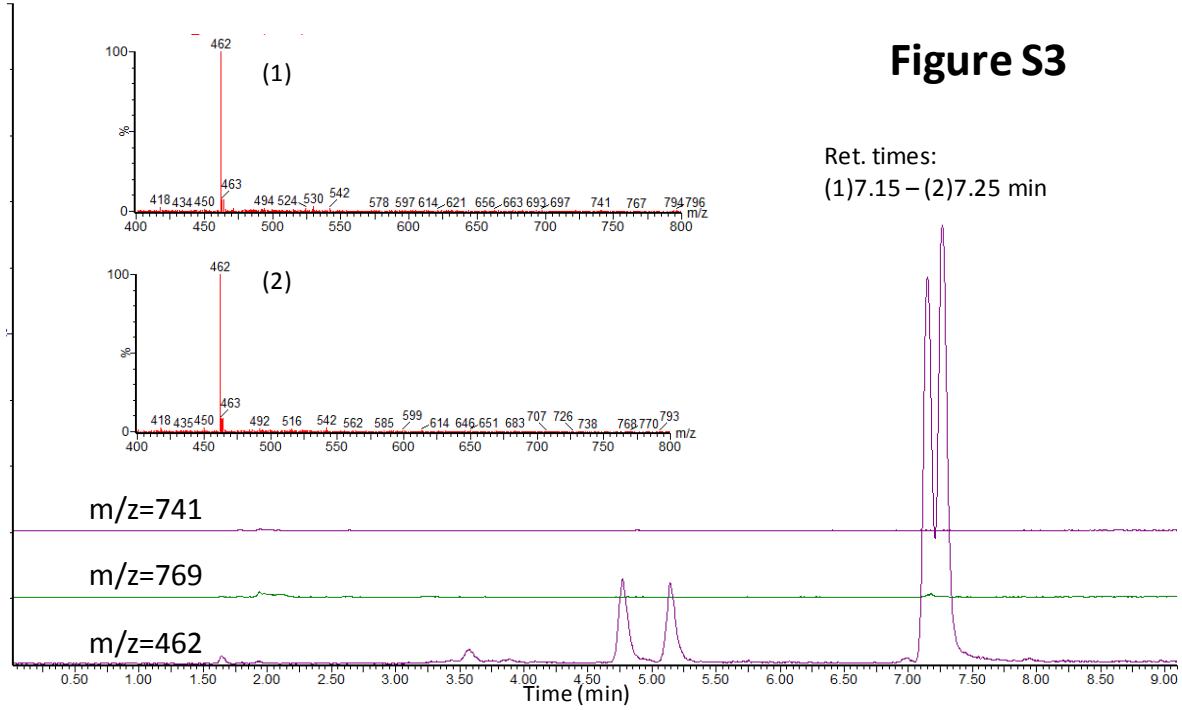

m/z=462
Sample pH 7.5 (1/100)
Sample pH 3.5 (1/1000)
Compound type C
SG
O
O
COOH
Ret. times:
(1)7.15 – (2)7.25 min
(1)
(2)
Time (min)

Figure S3



Ret. times:
(1)7.15 – (2)7.25 min

CRediT author statement

Dr. Juan José Rodríguez-Bencomo: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - review & editing. **Dr. Vicente Sanchis:** Conceptualization, Methodology, Resources, Writing - review & editing. **Dr. Inmaculada Viñas:** Conceptualization, Methodology, Resources, Writing - review & editing. **Dr. Olga Martín-Belloso:** Conceptualization, Methodology, Resources, Writing - review & editing. **Dr. Robert Soliva-Fortuny:** Conceptualization, Methodology, Resources, Writing - review & editing.